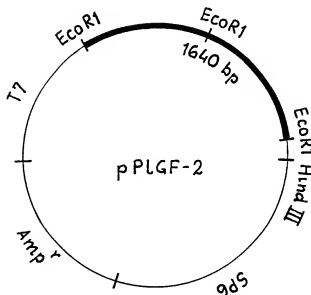




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification ⁵ : C12N 15/16, 15/62, C12P 21/02 C12N 1/21, 5/16 // (C12N 1/21 C12R 1/19) | A1 | (11) International Publication Number: WO 92/06194 (43) International Publication Date: 16 April 1992 (16.04.92) |
| (21) International Application Number: PCT/IT91/00079 (22) International Filing Date: 26 September 1991 (26.09.91) (30) Priority data: 48315 A/90 27 September 1990 (27.09.90) IT (71) Applicant (for all designated States except US): CONSIG- LIO NAZIONALE DELLE RICERCHE [IT/IT]; P.le Aldo Moro, 7, I-00185 Rome (IT). (72) Inventors; and (75) Inventors/Applicants (for US only) : PERSICO, Maria [IT/ IT]; MAGLIONE, Domenico [IT/IT]; I.I.G.B. C.N.R., V.le Marconi, 10, I-80125 Napoli (IT). (74) Agents: BANCHETTI, Marina et al.; Ing. Barzanò & Zan- ardo Roma S.p.A., Via Piemonte, 26, I-00187 Rome (IT). | | (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (Eu- ropean patent), GN (OAPI patent), GR (European pa- tent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European pa- tent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU*, TD (OAPI patent), TG (OAPI pa- tent), US. Published <i>With international search report.</i> |

(54) Title: NUCLEOTIDE SEQUENCES CODING FOR A HUMAN PROTEIN WITH ANGIOGENESIS REGULATIVE PROPERTIES

**(57) Abstract**

A cDNA sequence coding for a human protein having regulative properties of angiogenesis has been isolated and sequenced. Expression vector containing such sequence have been constructed and, after transformation of host cells, the synthesis of the related protein has been obtained. Such protein called PIGF can be employed both in the tumoral immunologic and diagnostic field and in the therapeutic field for pathologies related to the formation of vessels, as for instance the healing of wounds and so on.

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|-----------------|--------------------------|
| AT | Austria | ES | Spain | MG | Madagascar |
| AU | Australia | FI | Finland | ML | Mali |
| BB | Barbados | FR | France | MN | Mongolia |
| BE | Belgium | GA | Gabon | MR | Mauritania |
| BF | Burkina Faso | GB | United Kingdom | MW | Malawi |
| BG | Bulgaria | GN | Guinea | NL | Netherlands |
| BJ | Benin | GR | Greece | NO | Norway |
| BR | Brazil | HU | Hungary | PL | Poland |
| CA | Canada | IT | Italy | RO | Romania |
| CF | Central African Republic | JP | Japan | SD | Sudan |
| CG | Congo | KP | Democratic People's Republic of Korea | SE | Sweden |
| CH | Switzerland | KR | Republic of Korea | SN | Senegal |
| CI | Côte d'Ivoire | LJ | Liechtenstein | SU ⁺ | Soviet Union |
| CM | Cameroon | LK | Sri Lanka | TD | Chad |
| CS | Czechoslovakia | LU | Luxembourg | TG | Togo |
| DE | Germany | MC | Monaco | US | United States of America |
| DK | Denmark | | | | |

NUCLEOTIDE SEQUENCES CODING FOR A HUMAN PROTEIN
WITH ANGIOGENESIS REGULATIVE PROPERTIES

5 This invention relates to nucleotide sequences coding for a human protein having angiogenesis regulative properties.

10 More particularly, this invention relates to the isolation and to the molecular characterization of a gene coding for a new protein having the properties of an angiogenic factor which regulates in vivo the formation and/or the regeneration of the vertebrate blood vessel system, and it also relates to the protein itself. Moreover, this invention also refers to vectors containing such sequence or parts thereof, to prokaryotic and eukaryotic cells transformed with such vectors, and to the employment of such vectors and of such cells for the production of the protein and of corresponding polyclonal and/or monoclonal antibodies as well.

20 It is well known that growth factors are polypeptides, synthesized and secreted by mammalian cells, capable of acting not only on the proliferation, but also on the differentiation and morphogenesis of target cells. Indeed, it has been shown that some growth factors exert their action by regulating mechanisms such as chemotaxis, activation of inflammatory system cells and repairing of tissues (Whitman, M. and Melton, D. A., 25 1989, Annual Rev. Cell Biol., 5, 93-117).

Because of the similar phenotype between cultured growth factors stimulated and retrovirus transformed cells, it has been suggested that common mechanisms 30

control such phenomena. Indeed, the interaction between a growth factor and its own specific receptor indirectly activates gene activity regulative proteins, through intermediate reactions involving different protein-kinases. Many of the components of this metabolic chain have been identified as the cellular analogs of viral oncogenes, suggesting how oncoviruses could interfere with normal cellular processes.

Many growth factors have been identified up to the present time, the corresponding genes have been cloned, and such factors have been divided into groups, on the basis of similar activities and/or of sequence homologies; among them there is the family of angiogenic factors.

Angiogenesis, or the formation of vessels of the vascular system, is a complex process occurring during embryogenesis, wound healing and organ regeneration. Moreover, some pathologies like the growth of solid tumors, some retinopathies and rheumatoid arthritis induce an aberrant angiogenesis (Risau W., 1990, Progress in Growth Factor Research, 2, 71-79).

Angiogenesis in vivo is a multi-step process, two of them being represented by the migration and the proliferation of endothelial cells devoted to the formation of vessels.

In the most recent years, many angiogenic factors have been identified, and the corresponding genes cloned. Among them: angiogenin, subject-matter of the patent application PCT no. 8701372; the platelet-derived endothelial growth factor PD-ECGF (Ishigawa et al., 1989,

- 3 -

Nature, 338, 557); the human vascular permeability factor, VPF (Keck et al., 1989, Science 246, 1309), which was cloned also in the mouse with the denomination of vascular endothelial growth factor, VEGF (Leung et al., 1989, Science, 246, 1306); the growth factors for fibroblasts, i.e., the acid factor, a-FGF, and the basic factor, b-FGF, the transforming growth factors alpha, TGF-., and beta, TGF-..(Folkman and Klagsburn, 1987, Science, 235, 442).

Angiogenic factors have been divided into two groups, according to their way of action: either directly on the vascular endothelial cells, by stimulating motility or mitosis, or indirectly on cells producing growth factors acting on endothelial cells.

In vitro analysis have put into evidence that angiogenic factors exert different effects on the motility and on the proliferation of endothelial cells. Indeed, some of them stimulate just one of the two events, other ones stimulate both events, whereas others seem to be ineffective in vitro, and ,lastly, other ones show even an inhibiting activity of the endothelial cellular proliferation. Such data point out that the regulation of angiogenesis is a complex process mediated by different components, many of which have not been identified as yet.

Accordingly it is evident that there is the need for identifying and isolating new angiogenic factors capable of stimulating the migration and differentiation of endothelial cells, to be utilized both in the diagnostic field, as tumoral markers and for inflammatory

- 4 -

diseases, and in the therapeutical field, for topic or internal use, for instance in the treatment of wounds, of tissues after a surgical operation, of transplantation, of burns, ulcers, etc.. Such factors can be employed
5 successfully also in vitro, as growth-stimulating of cell cultures.

Moreover, DNA recombinant techniques allow such factors to be produced in suitable amounts, in short times and at remarkably low costs.

10 Indeed, there is an increasing need for identifying new specific tumoral markers because of uncertainties in tumor diagnosis. Moreover, recent methods for producing hybrid proteins (Fitzgerald D. and Pastan I., 1989, J. Natl. Cancer Inst. 81, 1455-1463) and/or conjugate
15 antibodies (Pearson, J. W. et al., 1989 Cancer Res. 49, 3562-3567) with toxic molecules, are giving promising results in the field of tumoral serotherapy, with an increasingly growing demand for new factors to test. Finally, many of angiogenic factor genes have been cloned
20 starting from tumoral cells, whereas a better applicability in the therapeutic field of genes coming from non-neoplastic material is evident.

Accordingly, this invention provides nucleotide sequences coding for a protein having a regulative
25 activity of angiogenesis, said sequences being obtained from non-neoplastic tissue; vectors containing said sequences; cells transformed by said vectors and producing protein having biologic and/or immunologic activities of a new angiogenic factor, as well as the
30 protein itself, to be employed in diagnostic and

therapeutic fields.

This invention also provides a procedure for obtaining the protein, or parts thereof, by recombinant techniques, as well as its use as an antigen for the production of the corresponding polyclonal or monoclonal antibodies.

Indeed, molecular probes comprising sequences coding for the angiogenic factor subject-matter of the present invention can be employed as markers in the diagnosis of pathologies related to the aberrant production thereof, as the case of some tumoral pathologies for other angiogenic factors.

Moreover, the protein which is another subject-matter of this invention can be employed in the treatment of inflammatory diseases, in the treatment of wounds, of tissues after surgical operations, of transplantation, of burns of ulcers and so on. Such factor can also be employed in vitro successfully, as growth stimulating of cell cultures.

Finally, DNA recombinant techniques employed in the present invention allow to produce the molecular probes and proteins described above in suitable amounts, in short times and at remarkably reduced costs.

The nucleotide and amino acids chains of this invention can be employed for diagnostic tests and for therapeutic purposes, both as directly derived from host cells and as after suitable modifications, for obtaining a better production for compositions.

Accordingly, the object of this invention consists in nucleotide sequences coding for a protein, named PlGF,

- 6 -

with immunogenic and/or biologic properties of an angiogenesis regulative factor, having the amino acids sequence of SEQ ID N1.

As another embodiment of the invention, the PlGF amino acid sequence derives from alternative splicings of the primary transcript, preferably at the nucleotide sequence shown in SEQ ID N2, most preferably giving rise to an amino acid insertion of 21 amino acids, whose sequence is shown in SEQ ID N2, at position 141-142 of the amino acid sequence shown in SEQ ID N1.

An object of this invention also consists in nucleotide sequences coding for the PlGF protein, lacking and/or substituted in one or more amino acids, preferably deleted from the amino acid 1 to the amino acid 31 of SEQ ID N1; the present invention also provides nucleotide sequences which are allelic derivatives of the sequence coding for SEQ ID N1, as well as nucleotide sequences that are complementary to those coding for SEQ ID N1.

Again according to the present invention the nucleotide sequence can be covalently bounded to a nucleotide sequence which can be translated into amino acid sequence by employing the same reading frame of the gene coding for PlGF, which preferably does not interfere with the angiogenesis regulative activity of PlGF, and which more preferably codes for a protein portion having toxic activity.

Accordingly, the object of this invention also consists in the nucleotide sequence of SEQ ID N1, even though the same is lacking and/or substituted in one or more nucleotides, coding at its coding part for the

- 7 -

protein PlGF, as in SEQ ID N1.

The present invention also provides nucleotide sequences hybridizing with SEQ ID N1, or parts thereof; nucleotide sequences obtained both through natural and synthetic or semisynthetic methods, by substitution, deletion, insertion and inversion mutations, either concerning single bases or multiple bases, of sequence described in SEQ ID N1, or parts thereof; and nucleotide sequences comprising sequences coding for a protein having immunogenic and/or biologic properties similar to those exhibited by the protein PlGF or parts thereof.

A further aspect of this invention relates to the protein PlGF having the sequence disclosed in SEQ ID N1, or parts thereof, obtained either by means of recombinant DNA techniques or isolated from biologic tissues. Said protein, or parts thereof which are immunologically active, can be employed as antigens for producing polyclonal and/or monoclonal antibodies.

The present invention also provides cloning and/or expression vectors, both prokaryotic and eukaryotic, comprising the nucleotide sequences subject-matter of the invention, sequences promoting transcription located upstream and, in general, a selective marker. Preferably, sequences promoting transcription in an inducible manner, can also be present and enhancers, polyadenylation signals and so on, as well.

Again an object of the present invention consists in prokaryotic and eukaryotic cells transformed by said vectors to be employed for producing the PlGF protein or parts thereof.

- 8 -

Just for illustrative and not for limitative purposes the present invention will be described in the following examples. In what follows reference will be made to the enclosed Figures wherein:

5 Figure 1 represents the restriction map of the recombinant λ GT11 phage, comprising the sub 32 fragment;

 Figure 2 represents a "Northern blot" experiment employing the sub 32 cDNA fragment;

10 Figure 3 represents the restriction map of the plasmid pP16F-2;

 Figure 4 represents an exemplificative scheme of subcloning of a fragment coding for a portion of the protein P16F in the expression vector pET3 (Novagen, Madison WI, USA);

15 Figure 5 represents a polyacrylamide gel electrophoresis of the protein P16F, said protein being obtained through the recombinant way.

Example 1

Isolation of the cDNA coding for a new angiogenic factor

20 A first cDNA fragment, named sub 32, was isolated from a clone of a cDNA library from human placenta, in the λ GT11 vector, according to conventional procedures and employed also in other laboratories (Watanabe et al., J. Biol. Chem. 264, 12611-19, 1989).

25 Briefly, RNA was extracted through lysis with guanidine thiocyanate and centrifuging on a discontinuous gradient of caesium (Sambrook J., Fritsch E.F., Maniatis T., Molecular Cloning - A Laboratory Manual. Second edition. Vol. 1, 7.19. Cold Spring Harbor Lab. Press).

30 The poly A+ RNA was purified through chromatography on

- 9 -

oligo-dT cellulose (ibid. 7.26). The cDNA synthesis and the cloning of the λ GT11 phage vector (Stratagene, La Jolla California, USA) in the Eco R1 restriction site was carried out following the protocol described ibid. 8.54-8.79. A clone, whose map is shown in Figure 1, was identified because of comprising also a sequence of 2600 nucleotides capable of hybridizing, in 5x SSC at 65C according to the hybridization procedure on filters described ibid. 8.46, with a sequence coding for the cDNA of the glucose-6-phosphate dehydrogenase enzyme (G6PD) (Persico, M. et al., 1986, Nucl. Acid Res., 14, 2511). A fragment of 240 nucleotides was also isolated from this recombinant phage after digestion with Eco R1 and Bam H1, and the fragment was called sub 32. Said fragment, after labelling with 32 P by means of the "nick translation" procedure disclosed ibid. 10.6-10.8 was employed for:

a) analyzing RNAs extracted from different tissues or cell lines, by "Northern blot" procedure as described in ibid. 7.37. The results shown in Figure 2 show that the sub 32 fragment detects specific mRNA in the placenta (line 2), in HEPG2 hepatoma cells, ATCC N. HB8065 (line 3), in JEG human choriocarcinoma cells, ATCC N. HTB36 (line 4) and, at lower concentration, in Hela S3 cells, ATCC N. CCL2.2 (line 5), but not in HL60 cells, ATCC N. CCL240 (line 1);

b) screening a cDNA library from JEG human choriocarcinoma JEG, ATCC N. HTB36, according to the procedures described for the cDNA library from human placenta, in the λ GT10 vector (Stratagene, La Jolla California, USA), in the Eco R1 site. Two clones were

- 10 -

isolated, digested with Eco R1 and subcloned in the pUC 18 vector (Stratagene, La Jolla, California, USA) and the sequence determined by Sanger's method (ibid. 13.6-13.10). The sequence revealed the fragments overlapped partially one another, but did not comprise the whole sequence coding for the corresponding mRNA. Hence, the isolated fragments were employed for a second screening, employing the same techniques. The library employed was the cDNA library from human placenta, from which the initial sub 32 fragment came from. Then two clones were isolated, their DNA was digested with Eco R1, the resulting inserts were subcloned in the pGEM 1 vector (Promega Corporation, Madison WI., USA) and their sequence was determined by Sanger's method. The two DNA fragments obtained after digestion with Eco R1 were religated together through T4-ligase and cloned in the same pGEM 1 vector in the Eco R1 site, to obtain the whole cDNA sequence corresponding to the mRNA present in the placenta, in a single plasmid, called pP1GF-2 (ATCC Dep. No. 40892), whose map is shown in Figure 3.

In order to confirm that the resulting fragment covers the whole coding sequence, the sequence was compared with the sequence of a genomic fragment obtained after hybridization of the same fragment with a genomic library from human fibroblasts WI38 (No. 944201 Stratagene, La Jolla, California, USA) in the λ Fix vector.

The cDNA sequence was identified according to Sanger's method (ibid. 13.3-13.10) and revealed:

a) a 5' end untranslated region of 321 nucleotides

- 11 -

comprising a sequence capable of forming a stem-loop
secondary structure, indicative of a translation
regulative signal;

b) a sequence of 447 nucleotides with an open
5 reading frame coding for a protein of 149 amino acids,
comprising a hydrophobic sequence of 32 amino acids at
the NH₂-terminal, indicative of the signal peptide of
secreted proteins;

c) a 3' end untranslated region of 877 nucleotides
10 comprising a polyadenylation site.

The amino acid sequence, deduced by the cDNA sequence, was inserted into the European Molecular Biology Laboratory (EMBL) Data Bank, showing no protein with the same sequence. A 50% homology, limited to a 120 amino acid region, was shown with the vascular permeability factor VPF (Keck et al., 1989, Science, 246, 1309), a powerful angiogenic factor, thus suggesting that the new protein PlGF can have by itself an angiogenesis-regulating activity.

20 Example 2

Screening of a cDNA library from JEG-3 cells with pPLGF
and structure of PlGF-gene

A cDNA library, obtained from JEG-3 cell mRNA, was screened with the P16F probe. Six recombinant phages were isolated. The sequence of two of them revealed they have a lenght of 510 bp, generating a 170 amino acid protein. The sequence resulted to be identical to the cDNA isolated from placenta, but an insertion of 63 bp, generating a 21 amino acid insertion into the protein, at position 141-142. Interestingly, the new sequence

contains 10 basic amino acids (Arg and Lys) over 21.

Example 3

genomic mapping and cloning of the PlGF gene

5 The gene coding for the protein PlGF was mapped on the chromosome 14 through "Southern blot" analysis, by employing DNA from different hybrid cellular lines, each containing different human chromosomes (not shown).

10 The structure and part of the nucleotide sequence of the PlGF gene was determined from a human genomic library. The gene is divided into six exons and five intervening sequences generating, through splicing, the transcript coding for the 149 aa. protein. In choriocarcinoma cells (JEG-3) the primary transcript is alternatively spliced at the fifth intron to generate a
15 transcript coding for the 170 aa. (see SEQ ID N2). Another alternative splicing involving the sequence from 174 to 828 of SEQ ID N2 of the fifth intron, gives rise to an higher molecular weight PlGF protein. In fact two proteins are immunoprecipitated from JEG-3 conditioned
20 medium, with antibodies anti PlGF.

Example 4

Subcloning of PlGF cDNA in a prokaryotic expression vector

25 A scheme of the subcloning strategy is shown in Figure 4, wherein the pET3 vector was employed (Novagen Madison Wi; USA) containing essentially the T7 phage RNA polymerase promoter, the terminator of the same phage, an origin of the replication (ori) and the resistance to ampicillin (amp).

30 The cDNA insert to be subcloned was obtained

through PCR amplification (polymerase chain reaction, *ibid.* 14.6), generating a cDNA coding for the protein lacking the first 31 amino acids. As template, the Eco RI DNA fragment, from nucleotide 1 to nucleotide 940, was employed. As primers for RNA polymerase the following oligonucleotides were employed, synthesized with an "Applied Biosystem 381A" oligo-synthesizer:

- oligonucleotide A complementary to the coding strand from the nucleotide 768 to the nucleotide 787, in which the GGATCC sequence, Bam HI recognition site, was inserted between nucleotides 775 and 776, having the following sequence:

5'-TCCTCCAAGGGGGATCCTGGGTTAC-3'

BamHI

- oligonucleotide B complementary to the non-coding strand from nucleotide 404 to nucleotide 421, in which the CATATG sequence, Nde I recognition site, was inserted between the nucleotides 414 and 415, having the following sequence:

3'-GCAAGGGGTATACTCGTCTGTTCC-5'

NdeI

The nucleotide chain, obtained from PCR, was digested with Nde I and Bam HI and ligated with the prokaryotic pET3 expression vector in the same Nde I and Bam HI sites according to standard protocols. The product was employed for transforming the E.coli HB101 strain which had been made competent with the CaCl₂ method. The recombinant plasmid was identified and employed for transforming the E.coli JM109 strain (DE3, Promega Corporation, Madison WI, USA).

Example 5

Synthesis and isolation of the PlGF protein from bacteria

A single colony was inoculated in LB broth containing 100 µg/ml of ampicillin (Sigma, St. Louis MO., USA) and 4 g/l of glucose and then grown at 37°C to reach an optical density O.D. of 0.35 at 600 nm. IPTG (Sigma) was added to a 1 mM final concentration and the culture was incubated at 37°C for additional 3 hours. The culture was centrifuged and resuspended in 1/10 of the initial volume of a buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE). Following further centrifugation, the precipitate was resuspended in 1/60 of the initial volume into a lysis buffer containing TE, 1% SDS, 0.1 M NaCl. Bacteria were divided into aliquots of 500 µl and subjected to lysis by three cycles of freezing and thawing, followed by middle strength sonication.

An example of the resulting electrophoretic pattern is shown in Figure 5, wherein lines 1, 2, 3 and 4 represent electrophoretic patterns of proteins from lysates respectively 0, 1, 2 and 3 hours after IPTG induction. As control, line 5 represents the same strain transformed only with the vector lacking the insert, induced with IPTG for 3 hours. Electrophoresis was carried out according to Laemmli, Nature, 227, 680-685, 1970, in a 15% polyacrylamide gel stained according to the method described by Bradley et al., Anal. Biochem. 182, 157-159 (1989).

Example 6

Production of anti PlGF antibodies and immunoprecipitations of PlGF

- 15 -

70 µg of the protein P16F was employed for immunizing two chickens, as described by Gassmann et al., 1990 Faseb J. 4, 2528-2532. The antibodies so formed were extracted and purified from the yolk through precipitation with polyethylene glycol (PEG) as described by Gassmann et al. (cf. above). The immunoprecipitations were performed by incubating 120-250 µl of cellular lysate, or Cos-1 cell conditioned medium, with 10 or 15 µl of rabbit or chicken antibodies, for 2 hours at room temperature, or 16 hours at 4°C. The immunoreactions with chicken antibodies were further treated with 15 µl of rabbit anti-chicken IgG (SIGMA N. C6778), for 1 hour at room temperature.

The immunocomplex was selected through protein-Sepharose 4B (Pharmacia) and washed twice with 1.2 µl of PBS with 0.01% Nonidet-P40 and 400 µM of NaCl.

The immunoprecipitates were then resuspended and analyzed on polyacrylamide gel under denaturing and reducing conditions according to standard procedure. If COS cells had been previously transfected with the plasmid pSVL-P16F, a protein of 25 KDa molecular weight is immunoprecipitated, both from the lysate and from the culture medium.

- 16 -

SPECIMEN SEQUENCE LISTING

SEQ ID N.1

SEQUENCE TYPE: nucleotide with corresponding
protein at the coding region

5 SEQUENCE LENGTH: 1645 bp

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

10 HYPOTHETICAL SEQUENCE: no

ANTI-SENSE: no

ORIGINAL SOURCE: cDNA placental library

ORGANISM: human

IMMEDIATE EXPERIMENTAL SOURCE: pP16F-2 (ATCC
N.40892)

15 FEATURES:

from 1 to 321 bp 5' end untranslated region

from 322 to 768 bp coding region

from 769 to 1645 bp 3' end untranslated region

PROPERTIES:

20 10 20 30 40 50 60
GGGATTTCGGGCCGCCAGCTACGGGAGGACCTGGAGTGGCACTGGGCGCCCGACGGACCA

 70 80 90 100 110 120
TCCCCGGGACCCGCTGCCCTCGGGCGCCCCGCCGGGGCCGCTCCCGTCGGGTTCT

25 130 140 150 160 170 180
CCCAGCCACAGCCTTACCTACGGGCTCCTGACTCCGCAAGGCTTCCAGAAGATGCTCGAA

 190 200 210 220 230 240
CCACCGGCCGGGGCCTCGGGGCAGCAGTGAGGGAGGCGTCCAGCCCCCACTCAGCTCTT

- 17 -

250 260 270 280 290 300
CTCCTCCTGTGCCAGGGGCTCCCCGGGGGATGAGCATGGTGGTTTTCCCTCGGAGCCCCC

310 320 330 340 350 360
TGGCTCGGGACGCTCTGAGAAGATGCCGGTCATGAGGCTGTTCCCTTGCTTCTGTCAGCTC
5 MetProValMetArgLeuPheProCysPheLeuGlnLeu

370 380 390 400 410 420
CTGGCCGGGCTGGCGCTGCCTGCTGTGCCCCCAGCAGTGGGCCTTGCTGCTGGGAAC
LeuAlaGlyLeuAlaLeuProAlaValProProGlnGlnTrpAlaLeuSerAlaGlyAsn

430 440 450 460 470 480
10 GGCTCGTCAGAGGTGGAAGTGGTACCCCTCCAGGAAGTGTGGGGCCGCAGCTACTGCCGG
GlySerSerGluValGluValValProPheGlnGluValTrpGlyArgSerTyrCysArg

490 500 510 520 530 540
GCGCTGGAGAGGCTGGTGGACGTCGTGTCGAGTACCCAGCGAGGTGGAGCACATGTTTC
AlaLeuGluArgLeuValAspValValSerGluTyrProSerGluValGluHisMetPhe

550 560 570 580 590 600
15 AGCCCATCCTGTGTCTCCCTGCTGCGCTGCACCGGCTGCTGCGGCGATGAGAATCTGCAC
SerProSerCysValSerLeuLeuArgCysThrGlyCysCysGlyAspGluAsnLeuHis

610 620 630 640 650 660
20 TGTGTGCGCGTGGAGACGGCCAATGTACCATGCAGCTCCTAAAGATCCGTTCTGGGGAC
CysValProValGluThrAlaAsnValThrMetGlnLeuLeuLysIleArgSerGlyAsp

670 680 690 700 710 720
CGGCCCTCCTACGTGGAGCTGACGTTCTCTCAGCACGTTGCTGCGAATGCCGGCCTCTG
ArgProSerTyrValGluLeuThrPheSerGlnHisValArgCysGluCysArgProLeu

730 740 750 760 770 780
25 CGGGAGAAGATGAAGCCGAAAGGTGCGGCGATGCTGTTCCCGGAGGTAAACCCACCCCT
ArgGluLysMetLysProGluArgCysGlyAspAlaValProArgArg

790 800 810 820 830 840
TGGAGGAGAGAGACCCCGACCCGCTCGTGTATTTATTACCGTCACACTCTTCAGTGAC

850 860 870 880 890 900
30 TCCTGTGTGTACCTGCCCTCTATTTATTAGCAACTGTTTCCCTGCTGAATGCCTCGCTC

910 920 930 940 950 960
CCTTCAAGACGAGGGGCAGGGAAGGACAGGACCCTCAGGAATTCAGTGCCTTCAACAACG

970 980 990 1000 1010 1020
TGAGAGAAAGAGAGAAGCCAGCCACAGACCCTGGGAGCTTCCGCTTTGAAAGAAGCAAG

1030 1040 1050 1060 1070 1080
ACACGTGGCCCTCGTGAGGGGCAAGCTAGGCCCCAGAGGCCCTGGAGGTCTCCAGGGGCT

1090 1100 1110 1120 1130 1140
GCAGAAGGAAAGAAGGGGCCCTGCTACCTGTTCTTGGGCCTCAGGCTCTGCACAGACAA

5 1150 1160 1170 1180 1190 1200
GCAGCCCTTGCTTTCGGAGCTCCTGTCCAAAGTAGGGATGCGGATTCTGCTGGGGCCGCC

1210 1220 1230 1240 1250 1260
ACGGCCTGGTGGTGGGAAGGCCGGCAGCGGGCGGAGGGGATTGAGCCACTTCCCCCTCTT

1270 1280 1290 1300 1310 1320
10 CTTCTGAAGATCAGAACATTGAGCTCTGGAGAACAGTGGTGGCTGGGGGCTTTTGCCAC

1330 1340 1350 1360 1370 1380
TCCTTGTCCCCCGTGATCTCCCTCACACTTTGCCATTTGCTTGTA CTGGGACATTGTTC

1390 1400 1410 1420 1430 1440
TTTCGGGCCGAGGTGCCACCACTGCCCCACTAAGAGACACATACAGATGGGGCCCCG

15 1450 1460 1470 1480 1490 1500
GGCTGGAGAAAGAGCTGCCTGGATGAGAAACAGCTCAGCCAGTGGGGATGAGGTCAACCA

1510 1520 1530 1540 1550 1560
GGGAGGAGCCTGTGCGTCCCAGCTGAAGGCAGTGGCAGGGGAGCAGGTCCCCAAGGGCC

1570 1580 1590 1600 1610 1620
20 CTGGCACCCCCACAAGCTGTCCCTGCAGGGCCATCTGACTGCCAAGCCAGATTCTCTTGA

1630 1640
ATAAAGTATTCTAGTGTGAAAACGC

- 19 -

SEQ ID N.2

SEQUENCE TYPE: nucleotide with the corresponding
aminoacid sequence for a reading frame

SEQUENCE LENGTH: 828 bp

5 STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

HYPOTHETICAL SEQUENCE: no

ANTI-SENSE: no

10 ORIGINAL SOURCE: genomic library

ORGANISM: human

IMMEDIATE EXPERIMENTAL SOURCE: plqfis5

FEATURES:

15 from 1 to 828 bp 5' end of the fifth
intervening sequence of the P16F gene
from 110 to 172 bp coding region due an
alternative splicing of the primary transcript
from 175 to 828 bp coding region due to another
alternative splicing of the primary transcript

20 PROPERTIES:

10 20 30 40 50 60
GTAAGTGGTTGGCTGGGGCTCGGGGCTATTCTATTCTCGGGCCTGCCAGCCTCTGTCCCT

70 80 90 100 110 120
AGCATGGGGTTCCCCAGCCACCTTGTCCTGACGCTTGGCTTATTGCAGGAGGAGACCCAA
ArgArgProLy

25 130 140 150 160 170 180
GGGCAGGGGGAAGAGGAGGAGAGAGAAGCAGAGACCCACAGACTGCCACCTGTGAGTGC
sGlyArgGlyLysArgArgArgGluLysGlnArgProThrAspCysHisLeu

- 20 -

190 200 210 220 230 240
CGGGGTCCCAGGGATGGCGAGGAGGCTGGGCCCAGGGGAGCCCCGCCTTGCCGCCAGGG

250 260 270 280 290 300
TTAGGTTGGGGAGGGGGAGAGGCAGGACTGAGGCCAGTCTTGGGG (G) CAGAACAGGGAN

5 310 320 330 340 350 360
CTGCACCTCCTCAAGACTCTAGGGCCCAGGAAGCATCAGTGGACCTTGCTTTTATCCCG

370 380 390 400 410 420
GCTTAGCCTAGGTTTCCATTGACCTTCAACAAATCATTTACCTTTGTCAGCCTAGCTTT

10 430 440 450 460 470 480
TCTCTGTGTAGAAATGAGGGGCAGGAGGTCCAGCAAACATTCAGTCACTCTACAAACATT

490 500 510 520 530 540
ACTGAGCACTTACTGTGTCTCAGGTACATCTGTGAGCAAACAAACAGGATTCTCTGCACAT

550 560 570 580 590 600
TAGTGTTTACCTTTTAGTGATTAAAAGTCTGTCTATCAGCTGAGACGTTATCTGGGGCCAC

15 610 620 630 640 650 660
TTCCTAGTAGCCCCGGGAACATGTGCCCTCNCACCTGTCTCCAGGAGTATTTTGCCTGT

670 680 690 700 710 720
GGGTCCCCCTTGCTGCTTCTAACCCACTTCGTACCTTGTGGGCAGCAGAATGGAGCCCCAG

20 730 740 750 760 770 780
GCCTGAGTGTGGCTGGGAGAGAAGGATGAGAGGAGGGAAAACCCAAATCTGTGAGAGTAA

790 800 810 820
ATAGAAAAAATAAAATATTTACGTGCACAGTCAATCAGTCACTGAAG

CLAIMS

1. A nucleotide sequence comprising a sequence coding for a protein with angiogenesis regulative activity having the sequence of SEQ ID N1.
- 5 2. A nucleotide sequence according to claim 1 wherein said coded protein derives from alternative splicings, different from those generating the sequence protein of SEQ ID N1.
- 10 3. A nucleotide sequence according to claim 2 wherein said alternative splicing generates a transcript coding for a protein with SEQ ID N1, with an insertion of 21 aa.. as in SEQ ID N2, at position 141-142 of SEQ ID N1.
- 15 4. A nucleotide sequence according to any of the previous claims, wherein said protein lacks one or more amino acids.
5. A nucleotide sequence according to any of the previous claims, wherein said protein has an altered regulative activity of angiogenesis.
- 20 6. A nucleotide sequence according to claims 4 or 5 wherein said protein is deleted from the amino acid 1 to the amino acid 31.
- 25 7. A nucleotide sequence according to any of claims 1 to 3 wherein one or more amino acids have been substituted in said protein.
8. A nucleotide sequence according to claim 7, wherein said protein shows an altered regulative activity of angiogenesis.
9. A nucleotide sequence according to any of the previous claims wherein said protein is an allelic

derivative.

10. A nucleotide sequence complementary to a sequence according to any of the previous claims.

5 11. A nucleotide sequence according to any of the previous claims, to which a nucleotide sequence, that can be translated with the same reading frame, has been covalently linked to the 5' or to the 3' positions.

10 12. A nucleotide sequence according to claim 11, wherein said linked nucleotide sequence codes for an amino acid sequence which does not interfere with the regulative activity of angiogenesis of the protein.

13. A nucleotide sequence according to claims 11 or 12 wherein said linked nucleotide sequence codes for a protein having a toxic activity.

15 14. A nucleotide sequence comprising the sequence of SEQ ID N1, wherein the 5' end region from the nucleotide 1 to the nucleotide 321 is untranslated, the region from the nucleotide 322 to the nucleotide 768 codes for a protein with angiogenesis regulative activity, the 3' end
20 region is untranslated.

15. A nucleotide sequence according to claim 14 comprising at least a part of sequence of SEQ ID N2.

25 16. A nucleotide sequence according to claim 14 or 15 lacking one or more nucleotides, coding for a protein having a regulative activity of angiogenesis.

17. A nucleotide sequence according to claims 14 or 15 lacking one or more nucleotides, coding for a protein having an altered regulative activity of angiogenesis.

30 18. A nucleotide sequence according to claims 16 or 17 which is deleted from the nucleotide 322 to the

- 23 -

nucleotide 414.

19. A nucleotide sequence according to claims 14 or 15 wherein one or more nucleotides have been substituted, coding for a protein having a regulative activity of angiogenesis.

20. A nucleotide sequence according to claims 14 or 15 wherein one or more nucleotides have been substituted, coding for a protein with an altered regulative activity of angiogenesis.

21. A nucleotide sequence according to claims 14 or 15, wherein said untranslated 5' region comprises a regulative region of the translation.

22. A nucleotide sequence according to claim 21, wherein said regulative region forms a stem-loop secondary structure.

23. A nucleotide sequence which is allelic to the nucleotide sequence according to claims 14 or 15.

24. A nucleotide sequence which is complementary to a nucleotide sequence according to any of the previous claims from 14 to 23.

25. A protein comprising the amino acid sequence of SEQ ID N1 having a regulative activity of angiogenesis.

26. A protein according to claim 25 wherein said protein comprises an insertion derived from alternative splicing of the primary transcript.

27. A protein according to claim 26 wherein said insertion is at position 141-142 of SEQ ID N1 and comprises a sequence coded by at least a portion of SEQ ID N2.

28. A protein according to claim 27 wherein said

sequence is of 21 amino acids as in SEQ ID N2.

29. A protein according to any of claims 22 from 25 to 28 wherein said protein lacks one or more amino acids.

5 30. A protein according to claim 29, wherein said protein shows an altered regulative activity of angiogenesis.

31. A protein according to claims 29 or 30, wherein said protein is deleted from the amino acid 1 to the amino acid 31.

10 32. A protein according to any of claims from 25 to 28 wherein one or more amino acids have been substituted in said protein.

33. A protein according to claim 32 wherein said protein shows an altered regulative activity of angiogenesis.

15 34. A protein coded by a nucleotide sequence which is an allelic derivative of the nucleotide sequence according to any of claims from 1 to 3.

20 35. A protein comprising an amino acid sequence according to anyone of the previous claims from 25 to 34, to which an amino acid chain is covalently linked at the terminal COOH or NH₂ groups.

25 36. A protein according to claim 35, wherein said amino acid chain does not interfere with the regulative activity of the angiogenesis of the protein itself.

37. A protein according to any of claims 35 or 36, wherein said amino acid chain shows a toxic cell activity.

30 38. A vector comprising: a) the replication origin of a bacterial plasmid, b) a selective marker, c) a

promoter and under the control of said promoter, d) the nucleotide sequence according to any of the previous claims from 1 to 24.

39. A vector according to claim 38, wherein said selective marker is a gene coding for an antibiotic resistance.

40. A vector according to any of claims 38 or 39, wherein said promoter is T7 phage RNA polymerase promoter.

41. A vector according to any of claims from 38 to 40 wherein said vector comprises the plasmid pGem 1.

42. A vector according to any of claims from 38 to 40 wherein said vector comprises the plasmid pET3.

43. A vector according to any of claims from 38 to 42 comprising one or more sequences that regulate the transcription.

44. A vector according to claim 43, wherein said sequences comprise a transcription enhancer.

45. A vector according to claim 43 wherein said sequences comprise an inducible promoter.

46. A vector according to any of claims from 38 to 45 comprising a polyadenylation site.

47. Cells transformed with a vector according to any of claims from 38 to 46.

48. Cells according to claim 47 wherein said cells are bacterial cells.

49. Cells according to claim 48 wherein said bacteria are E.coli.

50. Cells according to claim 47 wherein said cells are eukaryotic cells.

- 26 -

51. A process for the production and extraction of the protein PlGF from cells according to any of claims from 47 to 49, said process comprising: growing the bacterial culture in liquid medium to an optical density between 0.2 and resuspending in TE; centrifuging and resuspending in a lysis buffer; lysing the cells.

1/3

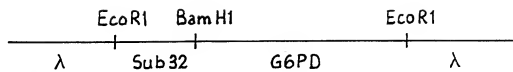


FIG. 1

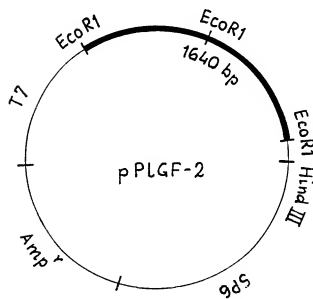


FIG. 3

2/3

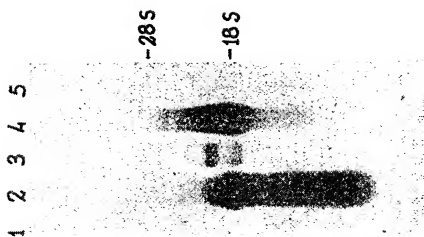


FIG. 2

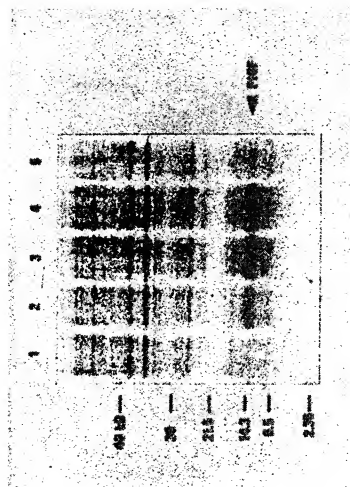
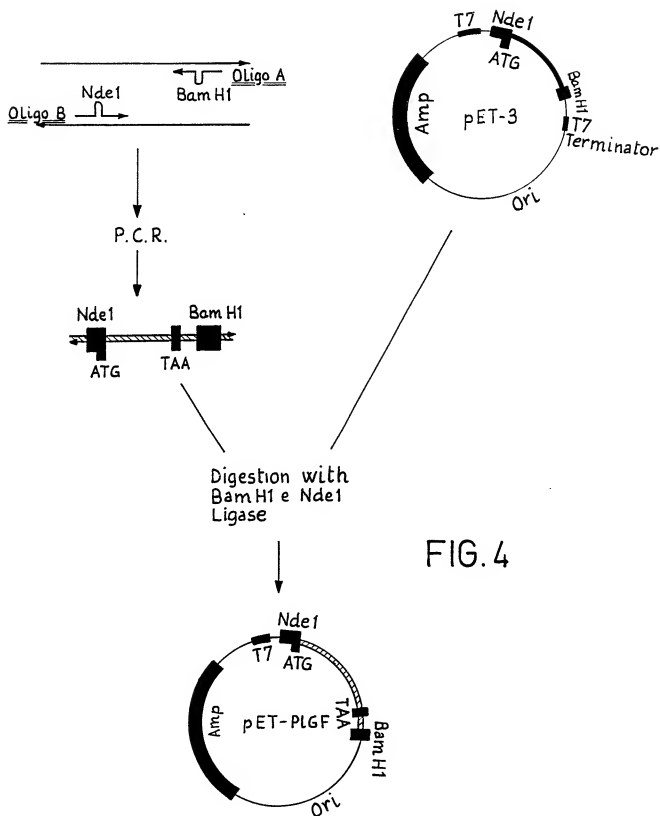


FIG. 5

3/3

CONSTRUCTION OF pET-PLGF



| | | |
|---|---|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁸ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| Int.Cl. 5 C12N15/16; C12N5/16; | C12N15/62; /(C12N1/21; C12R1:19) | C12P21/02; C12N1/21 |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁹ | | |
| Classification System | Classification Symbols | |
| Int.Cl. 5 | C07K | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ | | |
| | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ | | |
| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| P, X, O | J. CELL. BIOCHEM. SUPPL. 0 (15 PART F) 1991, NEW YORK, US; ABSTRACT NR. CF 316 page 242; M. G. PERSICO ET AL.: 'A NOVEL HUMAN ANGIOGENIC FACTOR PLGF' see the whole document & 'MEETING ON FGF, ENDOTHELIAL CELL GROWTH FACTORS AND ANGIOGENESIS HELD AT THE 20TH ANNUAL MEETING OF THE KEYSTONE SYMPOSIA ON MOLECULAR AND CELLULAR BIOLOGY, KEYSTONE, COLORADO, USA, APRIL 1-7 1991' --- | 1, 10, 14-16, 21-22, 24-25 |
| <p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | |
| 06 DECEMBER 1991 | 16.12.91 | |
| International Searching Authority | Signature of Authorized Officer | |
| EUROPEAN PATENT OFFICE | THIELE U.H. -C.H. <i>L. Kin</i> | |